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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Stationary-phase mutation is a mutational program that can be induced in non-dividing cells after exposure to environmental stress. We are testing the hypothesis that DNA double-strand breaks (DSBs) activate Lac^+ stationary-phase mutation in *E. coli*. In one model for stationary-phase mutation, recombination-mediated repair of a DSB is suggested to promote mutation by priming error-prone DNA replication. F plasmid transfer (Tra) proteins are required for stationary-phase reversion of a *lac* +1 frameshift mutation on the F'. Tra functions induce single-strand nicks on the F', which could lead to DSBs. We are asking whether DSBs introduced specifically near *lac* on the F' can substitute for Tra functions and activate stationary-phase mutation. To make specific DSBs, we constructed strains that express the *S. cerevisiae* endonuclease I-SceI from the *E. coli* chromosome. We find that introducing specific breaks at sites on either side of *lac* on a transfer-defective F' causes 50-2000-fold stimulations of Lac^+ stationary-phase mutation. This activation of mutation occurs only when both the I-SceI enzyme and cut site are present. The data imply that introduction of DSBs can overcome the requirement for Tra functions, and provide direct evidence that DSBs can activate stationary-phase mutation in the Lac system.

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Introduction:

Stationary-phase mutation, or adaptive mutation, refers to a collection of mutagenic responses that can be induced in stationary-phase (non-growing) cells after exposure to environmental stress. We would like to understand the processes that promote mutation in stationary-phase bacteria because similar mechanisms may be involved in mutation to antibiotic resistance in pathogens or genomic instability and cancer in higher eukaryotes. In the *E. coli* Lac system, cells carrying a chromosomal *lac* deletion and an F' sex plasmid with a *lac* +1 frameshift allele generate Lac^+ reversion mutants over time when starved on medium with lactose as the only carbon source. The mechanism for stationary-phase mutation is intrinsically different from that of growth-dependent mutation; it requires the homologous recombination proteins RecA, RecBCD, and RuvABC and the SOS-inducible, error-prone DNA polymerase, polIV. DNA double-strand breaks (DSBs) have also been implicated as molecular intermediates to stationary-phase mutation, although no direct evidence for DSB involvement has yet been obtained. In one model, recombination-mediated repair of a DSB is suggested to promote mutation by priming DNA replication using DNA polIV, during which polymerase errors occur. Cells carrying mutations that revert the *lac* +1 frameshift are able to utilize lactose in the medium and grow, escaping stress. We are asking whether DSBs can, in fact, activate Lac^+ stationary-phase mutation in *E. coli* and, if so, whether the mutation occurs in *cis* or in *trans* to the initiating DSB.

Body:

The goal of this project is to determine the role of DNA DSBs and DSB repair in Lac^+ recombination-dependent stationary-phase mutation in *E. coli*. Although stationary-phase mutation appears to occur throughout the genome, on the bacterial chromosome as well as the F' sex plasmid, the frequency of mutation varies widely from locus to locus. For example, the F' *lac* +1 framshift normally used in our assays mutates at a frequency of about $1x10^{-6}$ mutants per cell over the course of five days, whereas the frequency of mutation of a frameshift at the chromosomal *lac* locus is less than $1x10^{-8}$. We hypothesize that DSBs activate mutation in stationary-phase, and the rate of recombination-dependent mutation at a locus is directly affected by its proximity to DSBs.

DSBs could arise naturally in cells from DNA synthesis across an existing single-stranded nick, an induced enzymatic activity in stationary-phase, or an increased rate of oxidative damage (and its processing by endonucleases during repair). We hypothesize that the chromosomal *lac* locus has a low level of stationary-phase mutation because it lacks sufficient natural DSBs. In the case of the F', we know that plasmid-encoded transfer (Tra) proteins are required for stationary-phase mutation, although actual conjugative transfer is not. An endonuclease called TraI induces single-strand nicks at the origin of transfer on the F', and there are many ways in which a nick might become a DSB, such as a nick on the opposing DNA strand or passage of a replication fork. We

hypothesize that Tra proteins activate mutation on the F' because they promote DSBs by providing single-strand nicks.

We are asking whether DSBs introduced specifically near *lac* on the F' can 1) activate stationary-phase mutation and 2) substitute for Tra functions. To make specific DSBs, I constructed strains that express the *S. cerevisiae* endonuclease I-SceI (similar to yeast HO endonuclease) under the arabinose promoter, P_{BAD}, from *attB* in the *E. coli* chromosome. At the same time, I cloned the I-SceI restriction site, an 18bp sequence not present in the *E. coli* genome, into a defective miniTn*7*, hopped it throughout the genome, and isolated insertions to the left and to the right of *lac* on the F'. Once the desired hops were identified, I constructed strains that carry the specific I-SceI restriction sites on an F' deleted for TraI endonuclease and either the P_{BAD}-I-SceI gene or P_{BAD} alone at *attB* in the *E. coli* chromsome. These strains were actually constructed twice during the first six months of my fellowship due to a bacteriophage contamination problem we had in the lab. The reconstruction took nearly two months, but the new set of strains has shown no sign of contamination in multiple tests.

P_{BAD} is induced by arabinose and repressed by glucose, so we can control expression of I-SceI in our strains. However, if we plate the cells on arabinose and induce DSBs, death is observed (only) in strains carrying both the I-SceI gene and a cut site. In a the stationary-phase mutation assays I am doing, cultures of the strains to be tested are grown in minimal glycerol medium with 0.001% glucose added for repression of the arabinose promoter. Cultures are washed twice and plated on minimal lactose plates without arabinose, so any expression of the I-SceI endonuclease is driven by leaky expression from P_{BAD} in the absence of glucose. The number of Lac^+ colonies are then counted daily until five days after plating. Under these conditions, strains carrying both the I-SceI gene and a cut site still exhibit some death, such that the number of viable cells drops three- to five-fold over the course of five days.

In repeated sets of experiments, introduction of specific DSBs at two cut sites to the left of the *lac* +1 frameshift on a Tra-defective F' caused dramatic 2000-fold stimulations of Lac^+ stationary-phase mutation (figure 1). This effect was DSB-dependent because no increase in mutation was seen in any of the controls with enzyme but no cut site or cut site but no enzyme. Likewise, introduction of specific DSBs at two cut sites to the right of *lac* activated stationary-phase mutation on a Tra-defective F'; one cut site gave a 50-fold stimulation, while the other showed a 1000-fold effect (figure 2). These results provide the first direct evidence that DSBs can activate stationary-phase mutation and imply that the only role of Tra functions is to promote DSBs. The frequency of mutation in strains carrying both the I-SceI gene and a cut site has been variable from experiment to experiment and culture to culture, although the conclusions have remained unchanged. Using leaky, less-controlled expression from P_{BAD} is one likely source of the variability.

We would also like to know whether DSBs activate mutation by a *cis* or a *trans* mechanism. In the "*cis*" model for stationary-phase mutation I described above, a DSB that occurs near *lac* leads to an SOS DNA damage response and increased levels of DNA polIV. Repair of the DSB creates recombination intermediates that are proposed to prime error-prone DNA synthesis by DNA pol IV at *lac*. In this model, the initiating DSB, recombinational repair, and resulting mutation all occur in *cis* on the DNA. However, we could also draw a "*trans*" model for stationary-phase mutation which is initiated by a

DSB anywhere in the genome. In this model, a DSB leads to induction of the SOS response. Pol IV is upregulated during SOS, and makes polymerase errors in areas of DNA synthesis throughout the cell, in *trans* to the recombinational repair of the initiating DSB. If the *cis* model is correct, and the rate of recombination-dependent mutation at a locus is directly affected by its proximity to DSBs, then we should be able to increase mutation on a Tra-defective F' by inducing specific DSBs in *cis*, but not in *trans*, to the target. If the *trans* model is correct, then specific DSBs induced in *cis* and in *trans* to a target should both promote mutation.

I have already shown that specific DSBs in *cis* to *lac* can activate stationary-phase mutation on a Tra-defective F'. To test whether DSBs made in *trans* would also activate mutation, I created strains that carry either the P_{BAD}-I-SceI gene or P_{BAD} alone at *attB* and an I-SceI cut site at *upp* in the chromosome. In repeated sets of experiments, introduction of specific DSBs at *upp*, in *trans* to the *lac* +1 frameshift on a Tra-defective F', had no effect on Lac^+ stationary-phase mutation (figure 3). Unfortunately, we can not yet conclude that DSBs activate stationary-phase mutation by a *cis* mechanism because we have not yet been able to quantify the level of DSBs created at the various cut sites on the F' and the chromosome. We would also like to assay at least one other *trans* location on the chromosome and one on a third replicon where making DSBs would not affect cell viability, such as a plasmid.

All of the work described thus far has studied activation of stationary-phase mutation on a Tra-defective F'. I have also constructed a similar set of I-SceI strains to ask whether introduction of specific DSBs can activate reversion of a +1 frameshift at the chromosomal *lac* locus, a site notoriously cold for stationary-phase mutation. Experiments using these strains are currently underway. Please be aware that none of this material has been published, with the exception of the P_{BAD}-I-SceI allele construction.

Key Research Accomplishments (July 2000-July 2001):

- Gathered the first direct evidence that DSBs activate Lac^+ stationary-phase mutation
- Demonstrated that DSBs can substitue for transfer functions in stationary-phase mutation
- Mentored 3 students in projects dealing with mutation and recombination in *E. coli.*

Reportable Outcomes:

Publications:

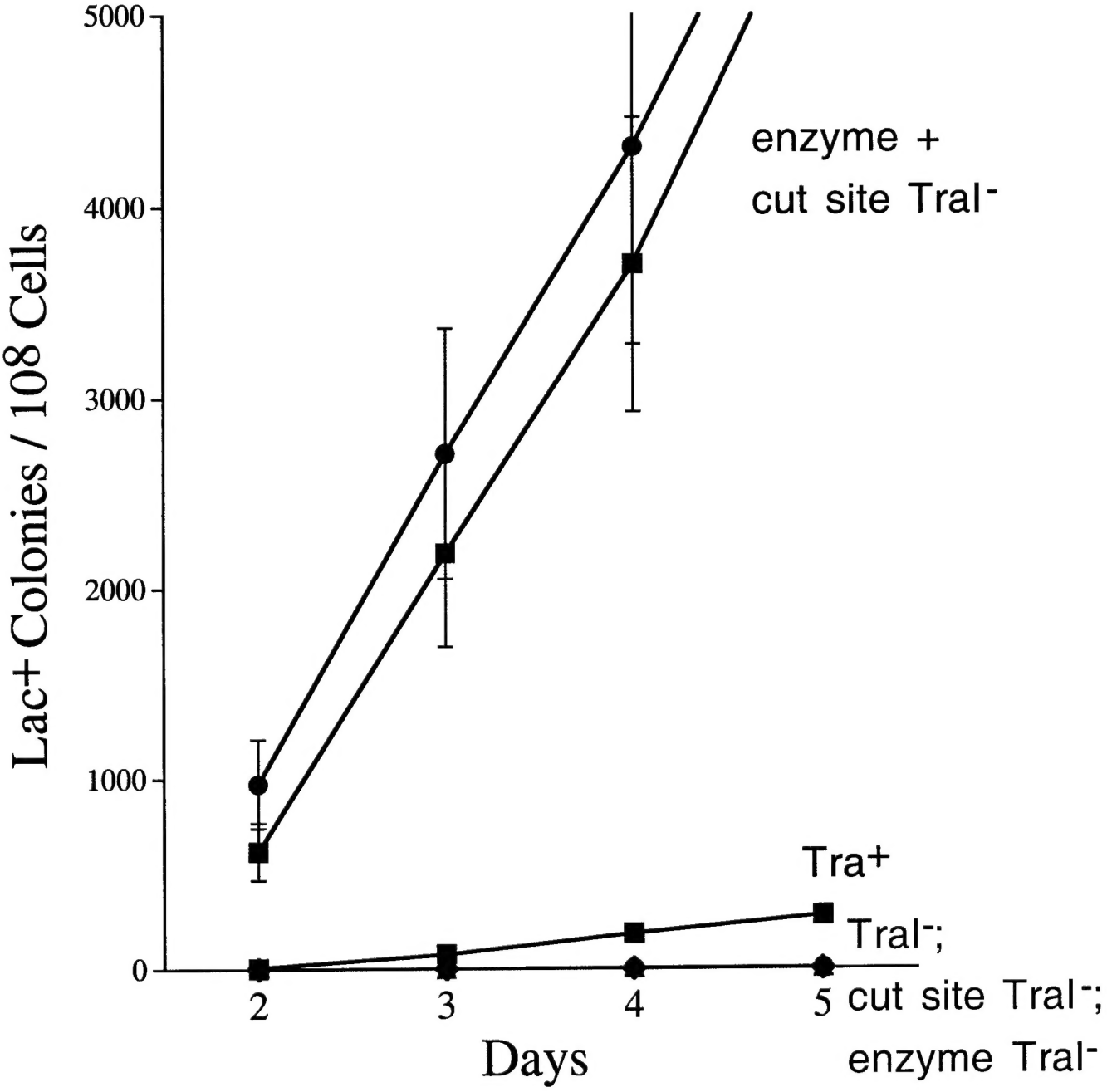
L. M. Gumbiner-Russo et al. 2001. The TGV *T*ransgenic *V*ectors for Single Copy Gene Expression from the *Escherichia coli* Chromosome. *Gene* 273: 97-104.

Conclusions:

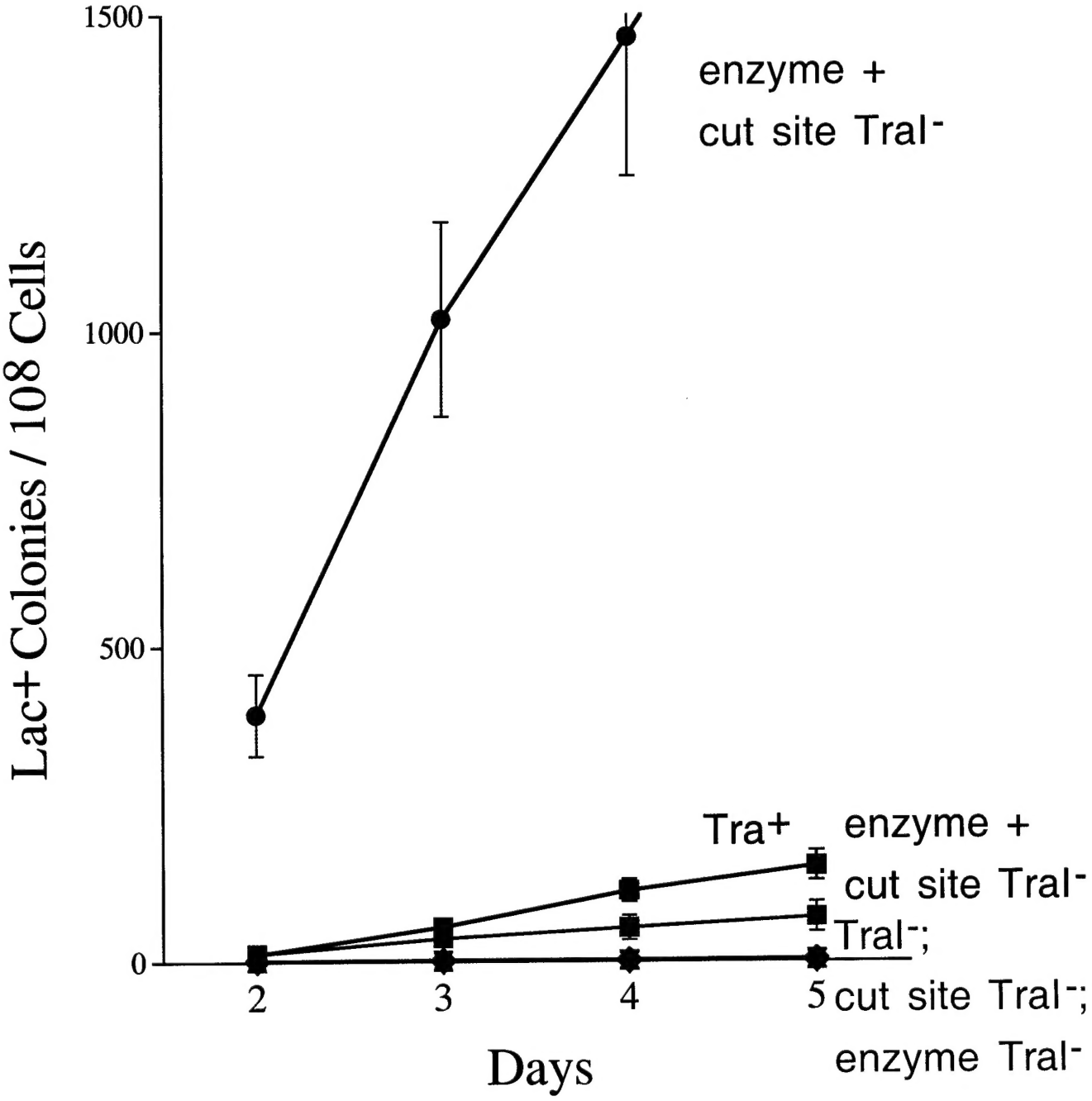
The mechanism for stationary-phase mutation requires the homologous recombination proteins RecA, RecBCD, and RuvABC and the SOS-inducible, error-prone DNA polymerase, polIV. Some of these prokaryotic DNA repair and mutation proteins are homologs of human DNA damage response proteins; RecA is a homolog of hRAD51, which associates with the DNA repair BRCA tumor suppressor proteins, and *E. coli* DNA polIV, or DinB, is a homolog of four new human DNA polymerases: RAD30a (the XPV tumor suppressor protein), RAD30b, REV1, and DINB1. The mechanisms by which these proteins act in environmentally-inducible mutation are likely relevant to cancer formation, progression, and resistance to chemotherapeutic drugs in humans.

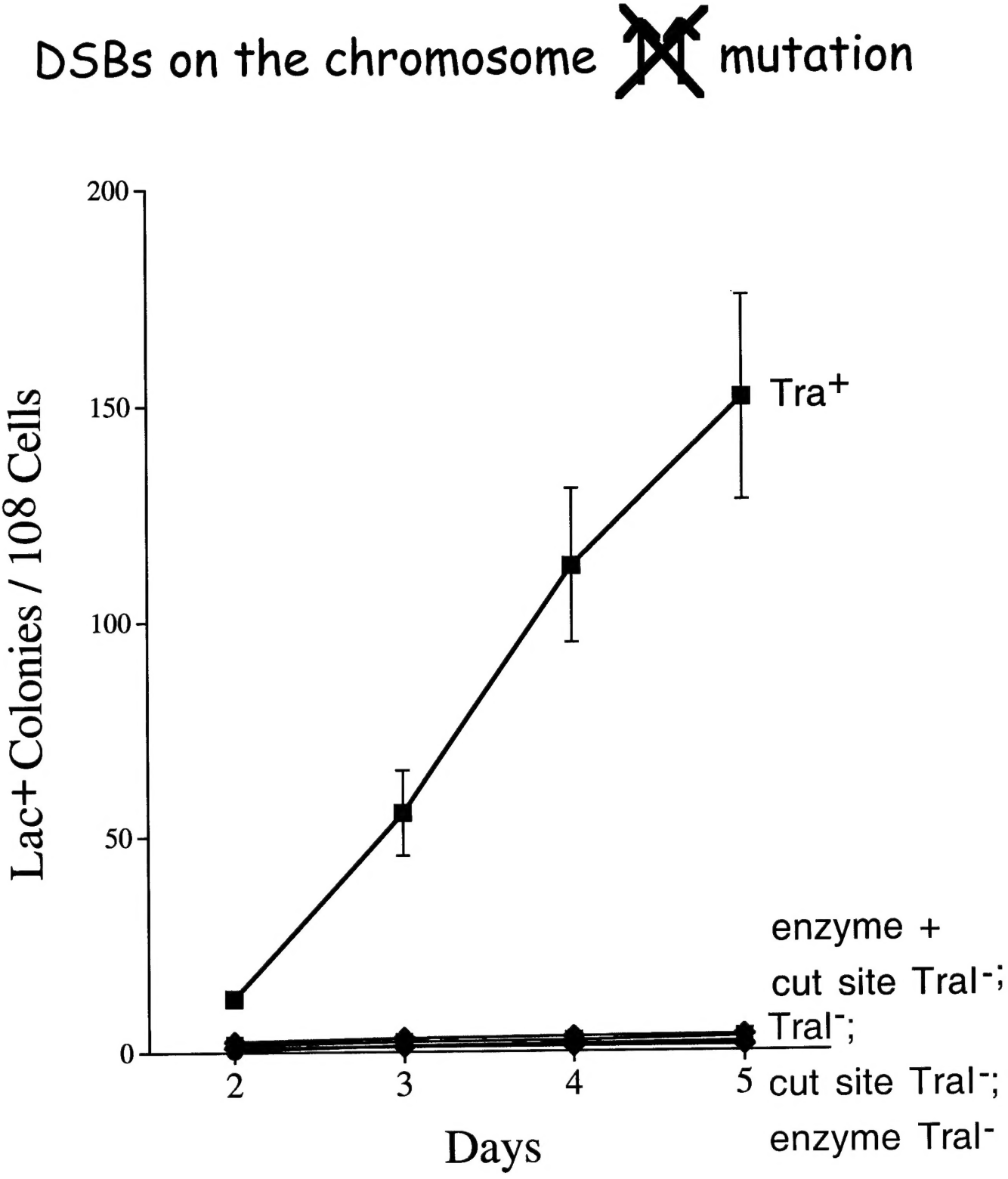
We find that introducing specific breaks at sites on either side of *lac* on a transfer-defective F' causes 50-2000-fold stimulations of *E. coli* Lac^+ stationary-phase mutation. This activation of mutation occurs only when both the I-SceI enzyme and cut site are present. The data imply that introduction of DSBs can overcome the requirement for Tra functions, and provide direct evidence that DSBs can activate stationary-phase mutation in the Lac system. Ongoing work in the lab is addressing whether DSBs activate mutation by a *cis* or a *trans* mechanism and asking whether the increased mutation occurs by a recombination-dependent, DNA polIV-dependent mechanism.

DSBs to the left of *lac* ↑↑ mutation



DSBs to the right of *lac* ↑↑ mutation




DSBs on the chromosome mutation
200
150
100
50
0
Lac+ Colonies / 108 Cells
2
3
4
5
Days
Tra+
enzyme +
cut site TraI-;
TraI-;
cut site TraI-;
enzyme TraI-